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THE EFFECT OF SALINITY ON GROWTH OF GYMNODINIUM BREVE DAVIS

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Field observations have established the close physical association of mass mortalities of marine animals in the Gulf of Mexico with high concentrations of the non-thecate dinoflagellate *Gymnodinium breve* Davis (Davis, 1948; Galtsoff, 1948, 1949; Gunter *et al.*, 1948; Wilson and Ray, 1956, among others). With the development of satisfactory culture media (Wilson and Collier, 1955) and successful methods for growing the organism in the absence of bacteria (Ray and Wilson, unpublished results), more definitive study of this association became possible. Subsequently Ray and Wilson (1957), and Starr (1958) conclusively demonstrated the toxicity of *G. breve* to fishes.

The catastrophic manifestations of naturally-occurring *G. breve* blooms have attracted considerable attention. The sporadic nature of the outbreaks has stimulated particular interest in possible relationships between environmental factors and these "red tides." In this regard, various investigators, drawing from relatively sparse field data, have postulated the importance of salinity, dissolved nutrients, and meteorological conditions (see Ryther, 1955, for review). This report deals with the effect of salinity on the growth *in vitro* of *G. breve* and compares these findings with the field observations of other workers.

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MATERIALS AND METHODS

Bacteria-free cultures (10-ml. aliquots in 16 × 125 mm. screw-capped Pyrex tubes) were employed throughout this work. These tubes, together with the flasks and pipettes used in medium preparation or inoculation, were rigorously cleaned before each use. The cleaning routine, found by Ray and Wilson (unpublished results) to be an important factor in the successful culturing of this organism, included the use of a detergent, hot 10% nitric acid, and repeated rinses in tap water and distilled water. In an additional step, culture tubes were filled with, and inoculation micropipettes immersed in, triple-distilled water and autoclaved for 15 minutes at 15 p.s.i.

All control tubes contained a completely synthetic medium (Table I), compounded by one of us (W.B.W.) which supported good growth of *G. breve*. The medium in experimental tubes differed from control medium only in major salt content (NaCl, $MgSO_4$, $MgCl_2$, $CaCl_2$ and KCl). In varying salinity these major constituents were varied proportionally, thus producing no change in the ion ratios.

Pasteur capillary pipettes were used to inoculate each of a series of tubes of medium with 100–200 cells from well-established cultures of *G. breve*. Two or three cultures were used to inoculate each experiment. Equal numbers of replicates from each salinity group were inoculated with a given culture so that physiological differences between inocula would not bias results. After inoculation, the new cultures were maintained at a temperature of 26–27° C., and illuminated by two 30-watt "standard cool white" fluorescent lights two to three inches from the culture tubes.

Glassware and media were sterilized by autoclaving at 15 p.s.i. for 15 minutes. After inoculations were completed, two bacterial sterility tests were conducted for each culture used as inoculum. These tests involved pipetting 1 ml. of the inoculum culture into each of two culture tubes, one containing 10 ml.

TABLE I

Gymnodinium breve *culture medium*

NaCl*	29.0 gm.	KNO_2	1.0 mg.
$MgSO_4 \cdot 7H_2O$	6.0 gm.	KNO_3	1.0 mg.
$MgCl_2 \cdot 6H_2O$	4.5 gm.	Thiamine†	1.0 mg.
$CaCl_2$	0.7 gm.	Vitamin B_{12}†	1.0 μg.
KCl	0.6 gm.	Biotin†	0.5 μg.
Tris(hydroxymethyl)-aminomethane**	20.0 mg.	Sulfide solution‡	5.0 ml.
$Na_2S \cdot 9H_2O$	3.0 mg.	Metals solution§	20.0 ml.
K_2HPO_4	1.0 mg.	Triple-distilled water	1000 ml.

* A. R. grade, recrystallized from triple-distilled water by the addition of C. P. HCl. All other inorganic compounds were C. P. or A. R. grade.

** Fisher Scientific Co. Added as 50 ml. of a stock solution adjusted to pH 8.2 by the addition of HCl.

† Nutritional Biochemicals Corp.

‡ Five ml. of this solution (derived from van Niel, 1931) contains: NH_4Cl, 1.0 mg.; $NaHCO_3$, 1.0 mg.; $Na_2S \cdot 9H_2O$, 0.8 mg.; KH_2PO_4, 0.5 mg.; $MgCl_2 \cdot 6H_2O$, 0.2 mg.

§ Twenty ml. of this solution contains: (Ethylenedinitrilo)tetraacetic acid disodium salt (Eastman Kodak Co.), 3.0 mg.; Mn as $MnCl_2 \cdot 4H_2O$, 0.2 mg.; Rb as RbCl, 0.2 mg.; Al as $AlCl_3 \cdot 6H_2O$, 0.1 mg.; Co as $CoCl_2 \cdot 6H_2O$, 0.1 mg.; Cs as CsCl, 0.1 mg.; B as H_3BO_3, 0.1 mg.; Se as H_2SeO_3, 0.1 mg.; Cr as K_2CrO_7, 0.1 mg.; Mo as $Na_2MoO_4 \cdot 2H_2O$, 0.1 mg.; Sr as $SrCl_2 \cdot 6H_2O$, 0.1 mg.; Ti as TiO_2, 0.1 mg.; Zn as $ZnCl_2$, 0.1 mg.; Zr as $ZrOCl_2 \cdot 8H_2O$, 0.1 mg.; Ba as $BaCl_2$, 0.02 mg.; Cd as $CdCl_2 \cdot 2\frac{1}{2}H_2O$, 0.02 mg.; Cu as $CuCl_2$, 0.02 mg.; Fe as $FeCl_2 \cdot 4H_2O$, 0.02 mg.; Ce as $(NH_4)_2Ce(NO_3)_6$, 0.02 mg.; V as NH_4VO_3, 0.02 mg.; Ni as $NiCl_2 \cdot 6H_2O$, 0.02 mg.; Rh as $RhCl_3$, 0.02 mg.; Ru as $RuCl_3$, 0.02 mg.; Sn as $SnCl_2 \cdot 2H_2O$, 0.02 mg.

of peptone sea water broth, the other a 10 ml. peptone sea water agar slant (Spencer, 1952).

Growth of the dinoflagellate was estimated by visual examination of the tubed cultures with the aid of a stereoscopic miscroscope, using 9× magnification for most cultures, and 18× or 27× when populations were low. Eleven graded population categories were adopted and "peak population" arbitrarily defined to include the top three. A rough calibration of this method was carried out by making estimates and actual cell counts from the same cultures, and comparing results. This check was conducted on four occasions, and, in all, 99 test cultures were examined by both methods. Cultures showing peak population by visual

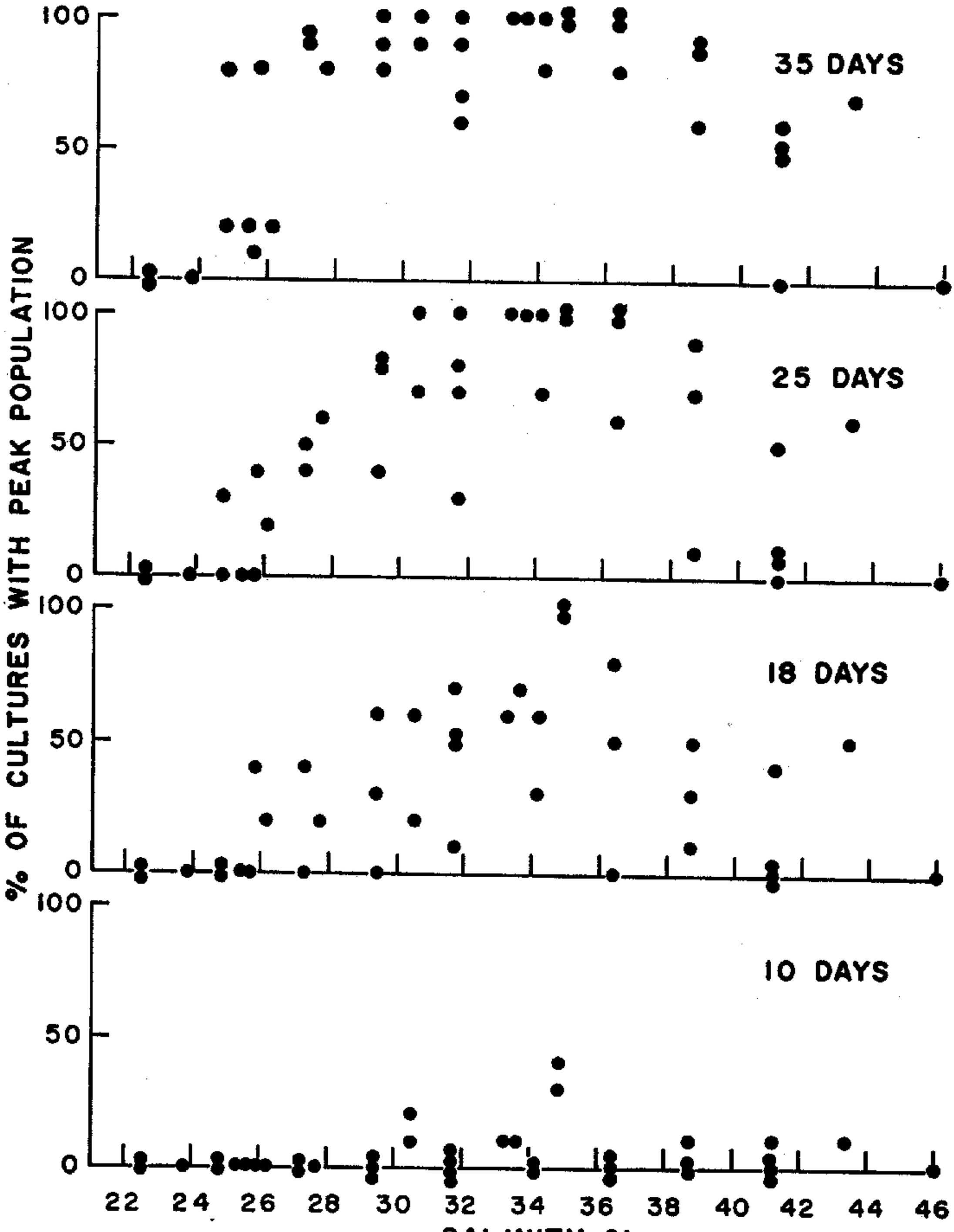

FIGURE 1. Rate of peak population development of *Gymnodinium breve* at various salinities. Each point is based on 10 replicate cultures.

estimate never proved to have fewer than 750 cells per ml., and usually contained from one thousand to several thousand cells per ml. Although sacrificing a degree of quantitative accuracy, the visual estimate method was selected because of the ease, speed, and lack of bacterial contamination with which it could be performed. All cultures were examined by this method at 4, 10, 18, 25, and 35 days after inoculation. The five-week period was considered adequate, since cultures seldom grow after five weeks post-inoculation.

Five experiments were conducted, each having nine salinity levels and ten replicate tubes at each level. The first experiment had the widest salinity range (6.3, 8.4, 11.9, 13.7, 17.8, 18.3, 25.8, 31.7, and 41.1‰). The four later experiments were designed for a salinity range of 22.5 to 41.1‰ with intervals of approximately 2.3‰. However, due to technical error, the last two experiments had ranges of 25.4 to 36.3‰ and 23.8 to 46.0‰ and somewhat irregular intervals.

Results

Cultures of *G. breve* grew well throughout a salinity range of 27 to 37‰ (Fig. 1). Within this range, at least 80% of replicate cultures reached populations of 750 cells or more per ml. during the five-week experimental period. More variable results and generally poorer growth occurred at salinity levels immediately adjacent to the optimal range. No instances of optimum growth occurred at less than 24‰ or more than 44‰. Some indication of comparative growth rates may also be obtained from Figure 1. It is apparent that cultures reach high populations more rapidly within the optimal range (27 to 37‰).

Some organisms survived throughout a salinity range of 22.5 to 46.0‰. From 24.8 to 46.0‰, 91% of cultures contained living cells at the end of the five-week observation period. There was no indication of reduced survival at the extremes of this range. Below this range the incidence of survival was lower; at 23.8‰ the organism survived in only one of 10 replicate cultures, and at 22.5‰ 10 of 20 replicates contained surviving cells. No instances of five-week survival were noted at any of the tested salinity levels below 22.5‰. At 18.3 and 17.8‰ three and two, respectively, of the 10 tubes in each group contained a few live organisms 10 days after inoculation, but no survivors were found eight days later. Media with salt concentrations of 13.7‰ or less contained no visible live *G. breve* one day after inoculation or thereafter.

Discussion

Reports discussing dinoflagellate salinity tolerances indicate a general euryhaline tendency for the group (Biecheler, 1952; Braarud, 1951; Braarud and Rossavik, 1951; Braarud and Pappas, 1951; Nordli, 1953; Provasoli *et al.*, 1954). Of the 10 forms studied by these workers, only one, *Peridinium balticum,* gave evidence of a stenohaline character (Provasoli *et al.*, 1954). Our results suggest that the range of salinity tolerated by *G. breve* is narrower than that of the dinoflagellates studied by other workers.

The literature relating to the occurrence of *G. breve* in the field contains a variety of statements concerning salinity. Fritsch (1956), in a general statement concerning Dinophyceae, noted (p. 664): "In the oceanic plankton the naked

types abound, while the neritic plankton is far richer in armoured forms." Commenting on salinities existing during the 1946–47 series of red tide outbreaks along the Florida coast, Gunter *et al.*, (1948) state (p. 320): "Comparing these results with those of normal sea water at various stations in south Florida . . . one may conclude that the . . . salinities are not abnormal." This statement is based largely on salinity values of 35.5 to 37.0‰.

Other workers report the occurrence of *G. breve* blooms in Florida waters having salinities below those associated with the open Gulf. Hela (1956) concluded that 31 to 34‰ represented the most favorable salinity range for blooms. Odum *et al.* (1956) found the organism most frequently in waters having salinities of 33 to 35‰. Observations of *G. breve* blooms by Ketchum and Keen (1948) and Chew (1953) include salinity values with ranges of 32.5 to 33.2‰ and 33.49 to 34.50‰, respectively.

Slobodkin (1953) suggested (p. 151), ". . . red tides require a discrete mass of water of relatively low salinity." He pointed out that the salinity difference

TABLE II

Salinity and incidence of G. breve *in Florida west coast waters (computed from Finucane and Dragovich, 1959)*

Salinity range (‰)	Samples collected	Samples positive for *G. breve*		Samples containing *G. breve* in lethal concentrations (250 or more/ml.)	
		Number	Per cent	Number	Per cent
39.00–40.99	107	0	0	0	0
37.00–38.99	1063	27	2.5	0	0
35.00–36.99	4010	397	9.9	15	0.4
33.00–34.99	1473	359	24.4	53	3.6
31.00–32.99	379	55	14.5	1	0.3
29.00–30.99	179	21	11.7	2	1.1
27.00–28.99	114	10	8.8	0	0
25.00–26.99	77	6	7.8	0	0
23.00–24.99	45	4	8.9	1	2.2
21.00–22.99	37	3	8.1	0	0
0.00–20.99	401	0	0	0	0

between a water mass and the surrounding water tends physically to preserve the identity of the mass. The inference was that the duration of water masses "physiologically suitable" for *G. breve* blooms determined the population levels which could be reached. On the other hand, Ryther (1955), reviewing red tide conditions as reported by Ketchum and Keen (1948) and Chew (1953), pointed out (p. 401) that ". . . where such measurements have been made, the salinity in patches of red water does not appear to be significantly lower than that of the surrounding, clear ocean water."

The obvious differences in the results and conclusions of these field studies are probably related to the small number of observations made in each case. Even so, the noted salinity values fall within the optimum range suggested by our culture studies.

The frequency of *G. breve* occurrence was determined for more than 7000 water samples of known salinity (Table II), using the field data of Finucane and Dragovich (1959). The incidence of the organism was significantly higher in salt concentrations lower (33.00 to 34.99‰) than those usually encountered in waters of the open Gulf (35.00 to 36.99‰). This observation was even more striking when only the potentially fish-killing population densities (approximately 250 or more cells per ml.) were considered.

Reduced salinity *per se* was apparently not a biologically essential factor for good growth of *G. breve* in culture. In the field the association of high incidence of this flagellate with salinities slightly below those of undiluted Gulf water may be due to the presence of dissolved nutrients in land drainage of the Florida west coast (Wilson and Collier, 1955). Water with salt *and* nutrient concentrations conducive to growth of dense populations of this organism may only occur in areas which receive nutrient-rich fresh water. The physical effect of salinity differences on the "life expectancy" of water masses as discussed by Slobodkin (1953) may represent another way in which salinity affects the population of *G. breve*. However, evidence is lacking on this point.

Finucane and Dragovich's (1959) data also show a few instances of the occurrence of *G. breve* at salinity levels between 21 and 25‰. The presence of the organism under these conditions is noteworthy, although the relatively few water samples in this range necessitate wide confidence limits for the per cent incidence values obtained. The long survival of this flagellate in culture at salinity levels inhibitory to growth suggests that it may also exist in the field long after salinity conditions have ceased to be favorable. Furthermore, salinity decreases in the field are probably more gradual than those of our experimental conditions, and may permit more acclimation.

In regard to *G. breve* distribution, our results indicate that high salinity may limit growth of this organism only in areas in which high evaporation with low runoff and mixing cause salt concentrations to rise well above offshore Gulf values. At the other end of the range, however, below 24‰ salinity may be a limiting factor in estuarine environments. Slobodkin (personal communication cited by Ryther, 1955) suggested that between outbreaks "seed populations of this organism are maintained in the brackish to freshwater regions of the Florida Everglades." In opposition to this view, our findings show that marine waters may present the most favorable environment for subsistence. Furthermore, recent field data (Finucane and Dragovich, 1959) indicate a lower incidence of this flagellate in Florida west coast embayments than in the Gulf during non-red tide periods.

When considered with nutritional requirements, the relatively stenohaline character of *G. breve* may explain the comparatively localized distribution of dense populations of this protist.

Summary

1. Bacteria-free *Gymnodinium breve* were exposed to media with salinity values ranging from 6.3 to 46.0‰; the best growth occurred between 27 and 37‰. These results indicate *G. breve* to be a relative stenohaline dinoflagellate.

2. Field evidence associates high incidence of dense populations with salinity levels a few parts per thousand below those of the offshore waters of the Gulf of Mexico. Our results suggest that this field distribution does not represent a salinity requirement *per se,* since salt concentrations equivalent to those of the open Gulf did not inhibit growth of this organism in culture.

3. No instances of optimal growth occurred in culture media with salinity levels of 24‰ or less. Under equivalent estuarine conditions salinity may be a limiting factor in the natural distribution of *G. breve.*

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